Review

Deconstructing the molecular portraits of breast cancer

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ABSTRACT

Breast cancer is a heterogeneous disease in terms of histology, therapeutic response, dissemination patterns to distant sites, and patient outcomes. Global gene expression analyses using high-throughput technologies have helped to explain much of this heterogeneity and provided important new classifications of cancer patients. In the last decade, genomic studies have established five breast cancer intrinsic subtypes (Luminal A, Luminal B, HER2-enriched, Claudin-low, Basal-like) and a Normal Breast-like group. In this review, we dissect the most recent data on this genomic classification of breast cancer with a special focus on the Claudin-low subtype, which appears enriched for mesenchymal and stem cell features. In addition, we discuss how the combination of standard clinical-pathological markers with the information provided by these genomic entities might help further understand the biological complexity of this disease, increase the efficacy of current and novel therapies, and ultimately improve outcomes for breast cancer patients.

1. Introduction

Implementation of screening/prevention programs and novel treatment strategies is decreasing breast cancer mortality (Jemal et al., 2009). However, more than 120,000 estimated deaths due to breast cancer are expected annually in the US and Europe combined (Jemal et al., 2009; La Vecchia et al., 2009). A plausible explanation for this scenario is, in part, that we still lack a complete enough picture of the biologic heterogeneity of breast cancers with respect to molecular alterations, treatment sensitivity, and cellular composition. Importantly, this complexity is not entirely reflected by the main clinical parameters (age, node status, tumor size, histological grade) and pathological markers (estrogen receptor [ER], progesterone receptor [PR] and human epidermal growth factor receptor 2 [HER2]), all of which are routinely used in the clinic to stratify patients for prognostic predictions and to select treatments.

Studies based on global gene expression analyses have provided additional insights into this complex scenario. During the last 10 years, four molecular 'intrinsic' subtypes of breast cancer (Luminal A, Luminal B, HER2-enriched, and Basal-like) and a Normal Breast-like group have been identified and intensively studied (Perou et al., 2000; Sorlie et al., 2001). Known as the 'intrinsic subtypes of breast cancer', these groups of tumors have revealed critical differences in incidence

(Millikan et al., 2008; Carey et al., 2006), survival (Perou et al., 2000; Sorlie et al., 2001; Cheang et al., 2009; Hu et al., 2006), and response to treatment (Prat et al., 2010; Nielsen et al., 2010; Parker et al., 2009b; Hugh et al., 2009; Carey et al., 2007; Rouzier et al., 2005a). Importantly, the information provided by the intrinsic subtypes complements and expands the information provided by classical clinical-pathological markers (Parker et al., 2009b).

As genomic studies evolve, further sub-classification of breast tumors into new molecular entities is expected to occur. For example, a new breast cancer intrinsic subtype, known as Claudin-low, has been recently identified in human tumors, in mouse tumors (Herschkowitz et al., 2007), and in a panel of breast cancer cell lines (Prat et al., 2010). Clinically, the majority of Claudin-low tumors are poor prognosis ER-negative (ER−), PR-negative (PR−), and HER2-negative (HER2−) (i.e. triple-negative) invasive ductal carcinomas with a high frequency of metaplastic and medullary differentiation. Preliminary data shows that they have a response rate to standard neoadjuvant chemotherapy that is intermediate between Basal-like and Luminal tumors (Prat et al., 2010). Furthermore, Claudin-low tumors are enriched with unique biologic properties linked to mammary stem cells (MaSCs) (Lim et al., 2009), a Core EMT signature (Taube et al., 2010), and show features of tumor initiating cells (TICs, also known as Cancer Stem Cells [CSCs]) (Creighton et al., 2009; Hennessy et al., 2009), the study of which is leading to the formulation of new hypothesis regarding the 'cell of origin' of the different subtypes of breast cancers.

In this review, we comprehensively deconstruct the molecular portraits of breast cancer in three steps. First, we describe the molecular features of the Claudin-low subtype in human tumors and cell lines. Second, we discuss the main clinical-pathological characteristics and treatment sensitivity of the intrinsic subtypes. Finally, we review the CSC hypothesis and the potential developmental origin of each intrinsic subtype.

2.   Molecular identification and characterization of the Claudin-low intrinsic subtype

In 2007, Herschkowitz et al. (2007) analyzed 232 human breast samples by semi-unsupervised hierarchical clustering and compared their gene expression profiles versus 108 mammary tumors from multiple genetically engineered mouse models. In this report, a potential new intrinsic subtype, apparent in both mouse and human data sets, was identified; this 'Claudin-low' subtype was characterized by the low expression of genes involved in tight junctions and cell–cell adhesion, including three different Claudin genes. Interestingly, most of the defining characteristics of the Claudin-low human tumors were conserved in several mouse models including 3 models with engineered BRCA1 and/or p53 deficiencies.

Recently, we have reported a more comprehensive characterization of this rare intrinsic subtype (Prat et al., 2010). As shown in Figure 1A, hierarchical clustering analysis of 320 human breast tumors and 17 normal breast samples using a ∼1900 gene intrinsic list (Parker et al., 2009b) places the Claudin-low group next to the Basal-like subtype indicating

that both tumor types share some gene expression features. These shared features include low expression of the HER2 and the luminal gene clusters, as well as the genes HER2, ESR1, GATA3 and the luminal keratins 8 and 18. However, two intrinsic gene clusters are uniquely expressed (or not expressed) in the Claudin-low subtype. One of these clusters is enriched with cell–cell adhesion proteins and is found to show low expression within Claudin-low tumors. Among the ∼20 genes that compose this cluster are claudin 3, 4, 7, cingulin and occludin that are involved in tight junctions, and E-cadherin that is a calcium dependent cell adhesion protein. Conversely, the other cluster, which is composed of ∼40 genes, is highly enriched with immune system response genes and is highly expressed in Claudin-low samples. Many of these genes are known to be expressed by T- and B-lymphoid cells (i.e. CD4 and CD79a), indicating high immune cell infiltration in this tumor subtype. However, the origin of other immune-related genes highly expressed in Claudin-low tumors, such as interleukin 6 or CXCL2 might be produced by the actual tumor cells, or immune cells, or both (see below).

Two additional intrinsic gene clusters have characteristic levels of expression in Claudin-low tumors. The proliferation gene cluster, which is usually highly expressed by poor outcome subtypes such as Basal-like, HER2-enriched and Luminal B tumors, is expressed at low levels in almost all Claudin-low samples, although it does not reach the low levels observed in the Luminal A or Normal Breast-like groups (Prat et al., 2010). This data suggests that Claudin-low tumors might be slow cycling tumors. Conversely, a cluster composed of ∼80 genes and highly enriched with mesenchymal/extracellular matrix genes (i.e. laminin and integrin alpha 7) is highly expressed in Claudin-low tumors with a pattern of expression similar to the Normal Breast-like group, which is not unexpected since normal breast samples are highly enriched with stromal tissue.

It is important to note that the Normal Breast-like group in our studies has always contained many true normal breast samples (typically reduction Mammoplasty samples), with the PAM50 Normal Breast-like group being trained using only true normals (Parker et al., 2009b). The small number of tumors that fall into the Normal Breast-like group with the true normals, show low tumor cellularity (<50%) when examined pathologically, which likely explains why they group with the true normal samples (Parker et al., 2009b; Peppercorn et al., 2008). Other studies have questioned the existence of this subtype based upon analyses of data sets that typical contain no true normal samples, which depending upon the rigor of pathology quality control over % tumor cellularity, may result in only a handful of tumor samples showing this phenotype; thus, those proposing to assess the existence of this group must include true normal samples in their analyses, and when a tumor sample falls into this group it does so mostly likely because that sample is predominant composed of normal breast tissue and not tumor tissue. However, future larger studies such as The Cancer Genome Atlas project will address the existence of a normal-like breast tumor subtype.

The pattern of expression of the main intrinsic gene clusters across the molecular portraits of breast cancers reveals that the Claudin-low tumors are characterized by two main
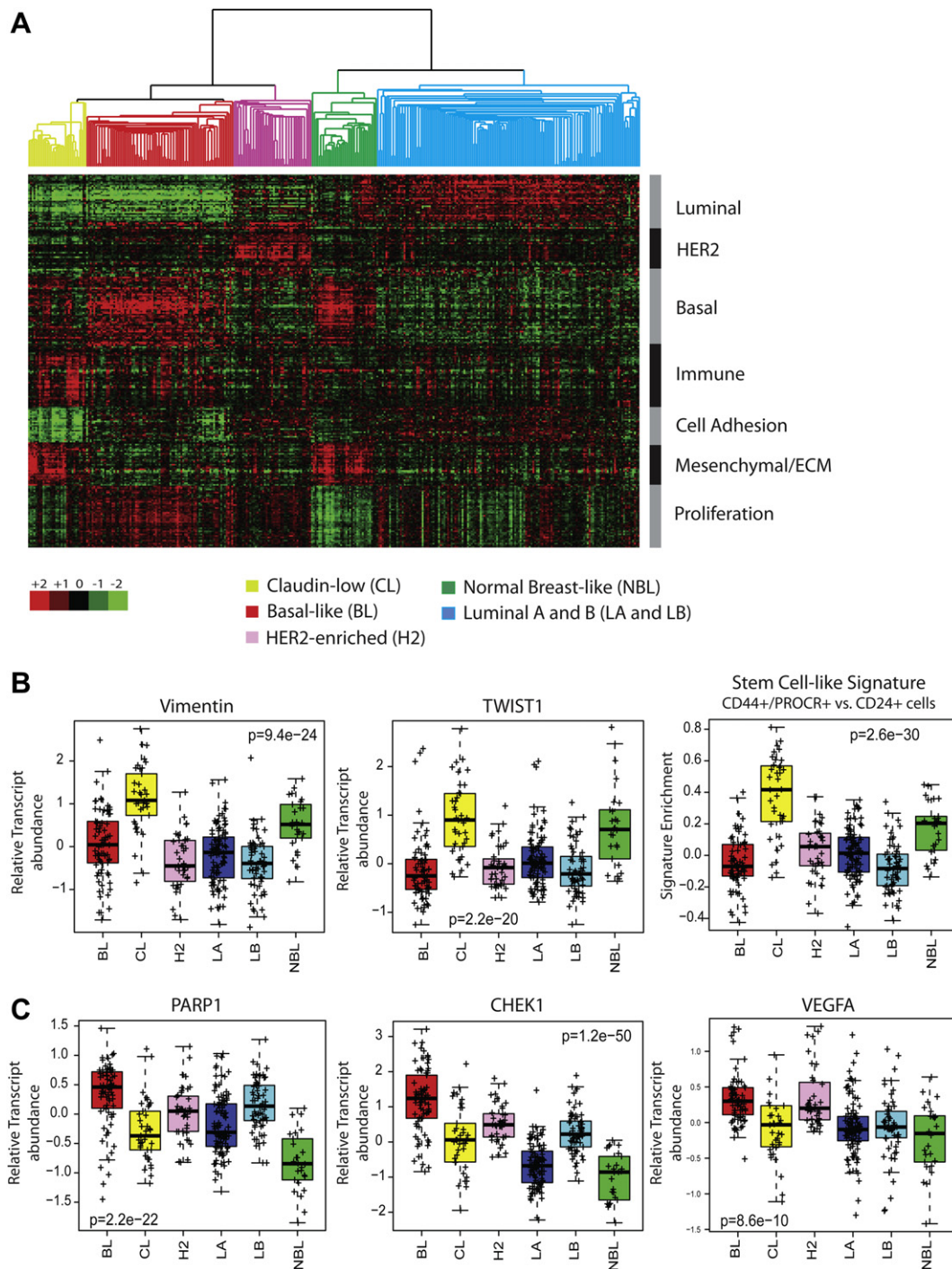
Figure 1 – Intrinsic hierarchical clustering and selected gene expression patterns of 337 UNC breast samples data set (publicly available at GSE18229 and https://genome.unc.edu). (A) Average-linkage hierarchical clustering of genes and arrays was performed using the intrinsic gene list from Parker et al. (2009b), with the sample associated dendrogram colored according to intrinsic subtype. Characteristic expression patterns are highlighted including the Luminal, HER2, Basal, Immune, Cell adhesion, Mesenchymal/Extracellular matrix (ECM) and Proliferation gene clusters. Each colored square represents the relative transcript abundance (in log2 space) with highest expression being red, average expression being black, and lowest expression being green. (B) Mesenchymal and stem cell-like gene expression in Claudin-low tumors shown using ANOVA analysis for each subtype. The Stem Cell-like Signature (CD44$^+$/PROCR$^+$ vs. CD24$^+$) was obtained from Shipitsin et al. (2007), and a enrichment/activity score was derived by calculating the inner product of this signature (gene ratio) and the gene expression value of each tumor sample. (C) DNA-repair (PARP1 and CHEK1) and angiogenesis (VEGFA) gene expression for individual genes across the subtypes.

features: high mesenchymal features and low luminal/epithelial differentiation. Interestingly, previous reports have linked both of these features by showing that the induction of a mesenchymal state in a mammary epithelial cell (also known as epithelial-to-mesenchymal transition [EMT]) is associated with the acquisition of undifferentiated mammary stem cell-like features (Taube et al., 2010; Morel et al., 2008; Gupta et al., 2009; Shipitsin et al., 2007; Mani et al., 2008). For example, expression of EMT-inducing transcription factors like SNAI1 (Mani et al., 2008), or repression of E-cadherin (Gupta et al., 2009) in mammary epithelial cells, causes a fibroblast-like appearance with induction of mesenchymal markers such as N-cadherin and/or vimentin. In addition, cells in this EMT state acquire a $CD44^+/CD24^{-/low}$ stem cell-like antigenic phenotype (Gupta et al., 2009; Al-Hajj et al., 2003), which has been previously found to enrich for CSCs (Gupta et al., 2009; Al-Hajj et al., 2003; Li et al., 2008). Indeed, EMT-inducing transcription factors such as ZEB2 and TWIST2, as well as the mesenchymal marker vimentin, are expressed at higher levels in $CD44^+CD24^{-/low}$ CSCs and $CD49f^{high}/EpCAM^-$ mammary stem cells (MaSCs) relative to the more differentiated $CD44^-/CD24^+$ tumor cells and $CD49f^-/EpCAM^+$ mature luminal cells, respectively (Shipitsin et al., 2007; Prat and Perou, 2009).

Concordant with a mesenchymal/stem cell-like state, Claudin-low tumors show the highest gene expression of vimentin and N-cadherin, and several known transcriptional repressors of E-cadherin (i.e. TWIST1) compared to the Basal-like and other tumor subtypes (Prat et al., 2010) (Figure 1B); in these Claudin-low tumors, it appears as if the vimentin is expressed within the stroma/fibroblasts and epithelial cells as revealed by dual label immuno-fluorescence experiments (Figure 2A−C). In addition, Claudin-low tumors show the lowest gene expression of epithelial differentiation markers such as CD24, EpCAM and MUC1, while showing higher expression of CD44 and CD49f (ITGA6) than luminal umors, which is concordant with $CD44^+/CD24^{-/low}$ and $CD49f^+/EpCAM^-$ stem cell-like antigenic phenotypes (Prat et al., 2010). Furthermore, various genomic signatures derived from either $CD44^+/CD24^{-/low}$ or normal breast MaSCs-enriched FAC sorted populations, have been found exclusively highly expressed within Claudin-low tumors (Prat et al., 2010; Creighton et al., 2009; Shipitsin et al., 2007; Dontu et al., 2003) (Stem Cell-like Signature, Figure 1B) and true normal breast specimens.

Another extensively studied stem cell/TIC/CSC marker, aldehyde dehydrogenase 1 (ALDH1) (Resetkova et al., 2010; Ginestier et al., 2007), is found highly expressed in both the Normal Breast-like group and Claudin-low tumors. This is not surprising since the expression of ALDH1 is not restricted to epithelial cells but also noted in stromal cells (Lim et al., 2009; Resetkova et al., 2010; Ginestier et al., 2007). Thus, whether the high gene expression of ALDH1 observed in
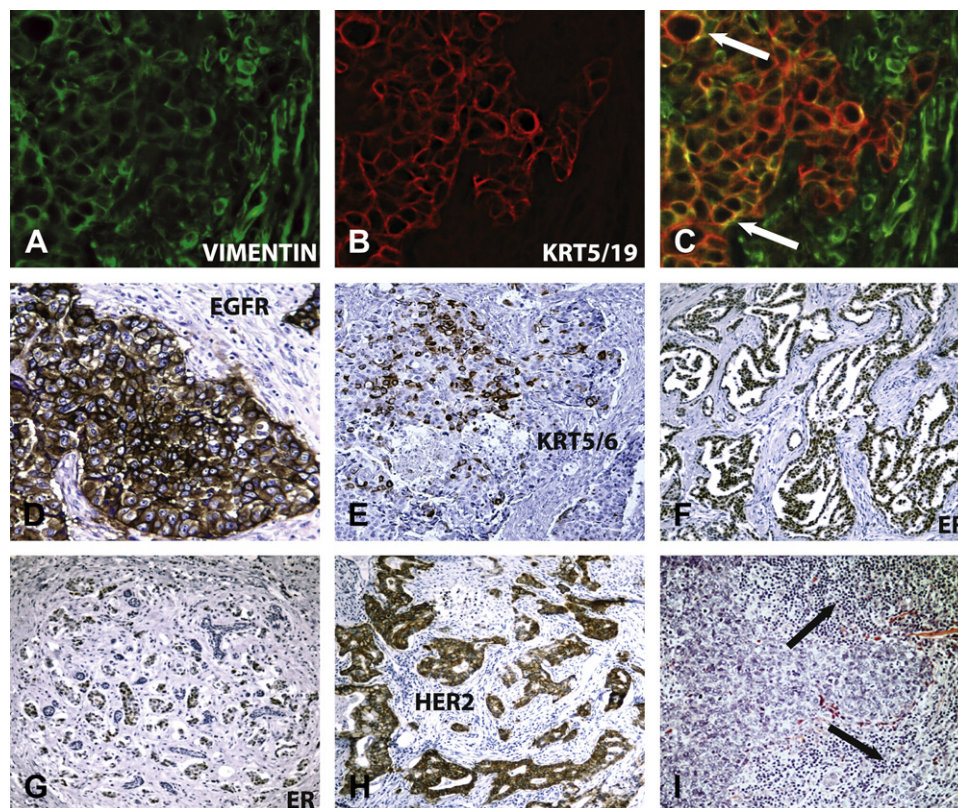
Figure 2 − Selected histological features of the intrinsic subtypes of breast cancer. (A−C) Dual label immuno-fluorescence analysis of a Claudin-low sample with tumor cells positive for vimentin (green, A), keratin 5/19 (red, B) and both (yellow, white arrows, C). (D) Basal-like sample with EGFR/HER1 positive staining. (E) Basal-like sample with keratin 5/6 positive staining. (F) Well-differentiated Luminal A tumor with strong ER-positivity. (G) Poorly differentiated Luminal B tumor with weak/moderate ER-positivity. (H) HER2-enriched tumor with strong membrane staining for HER2. (I) Claudin-low tumor with brisk lymphocytic infiltration (black arrows). This figure has been modified from Prat et al. (2010).

Claudin-low tumors has an origin in the stromal cells, the tumor cells, or both, is currently unknown but is under investigation. Overall, the molecular characterization of Claudin-low tumors suggests that breast epithelial cancer cells within this tumor subtype lack luminal and common epithelial cell features and are enriched with stem cell-like/mesenchymal characteristics that eventually attract stromal and/or immune-related cells into the microenvironment.

3.   Molecular identification of Claudin-low *in vitro* model systems

Previous studies have shown that the genetic and transcriptional characteristics of breast tumors are present in cell lines (Neve et al., 2006; Chin et al., 2006). In 2006, Neve et al. (2006) analyzed the expression pattern of 51 breast cancer cell lines and compared their profile with 145 primary breast tumors. Hierarchical clustering of the transcriptional profiles of these cell lines revealed two major clusters: luminal and basal. The luminal cluster included the majority of ER+ and/or HER2+ cell lines, while the basal cluster was further subdivided in two subgroups: Basal-A (BT20, HCC1143, HCC1187, HCC1569, HCC1937, HCC1954, HCC2157, HCC3153, HCC70, MDA-MB468, SUM190PT, and SUM225) and Basal-B (BT549, HBL100, HCC1500, HCC38, Hs578T, MDA-MB157, MDA-MB231, MDA-MB435, MDA-MB436, SUM1315, SUM149PT, and SUM159PT). Basal-A cell lines matched closely to the Basal-like signature found in primary tumors. Basal-B cell lines exhibited a profile that was less similar to *in vivo* Basal-like tumors, yet were still referred to as "Basal"; other investigators have used different names for these Basal-B cell lines such as Normal-like (Hollestelle et al., 2009; Sieuwerts et al., 2009). Interestingly, the Luminal, Basal-A and Basal-B molecular groups are also maintained during 3D cell culture (Kenny et al., 2007).

More recently, we have shown that 9 previously called "Basal-B/Normal-like" cell lines (BT549, HBL100, Hs578T, MDA-MB157, MDA-MB231, MDA-MB435, MDA-MB436, SUM1315, SUM159PT) most resemble the Claudin-low subtype (Prat et al., 2010). Among them, the triple-negative MDA-MB231 is one of the most widely used breast cancer cell line in cancer research due to its plasticity, invasive phenotype and high metastatic potential (Minn et al., 2005; Kim et al., 2009). Hierarchically clustering the gene expression data of Neve et al. (2006) with an intrinsic gene list reveals that these 9-cell lines cluster together and display similar gene expression patterns as Claudin-low human tumors, namely low expression of the luminal and HER2 gene clusters, inconsistent expression of the basal cluster, and low expression of the cell–cell adhesion cluster containing claudin 3, 4 and 7, and E-cadherin (Figure 3A). Importantly, the top upregulated and downregulated genes found in these 9 breast cancer cell lines, when compared versus all other cell lines, were found similarly expressed in Claudin-low tumors (Figure 3B). A major difference, however, is that Claudin-low cell lines do not show low expression of the proliferation cluster as do the *in vivo* Claudin-low tumors; a potential explanation of this finding is unknown but might be secondary to the *in vitro* culture conditions and/or selection process where slow growing cells are selected against.

As with Claudin-low tumors, accumulating evidence suggest that these Claudin-low cell lines are enriched with stem cell-like features. For example, Charafe-Jauffret et al. (2009) reported that many of these cell lines show high expression of ALDH1 and contain functional CSCs. This is in concordance with two other reports (Fillmore and Kuperwasser, 2008; Sheridan et al., 2006) that showed that the MDA-MB231, SUM159PT, SUM1315 MDA-MB436, Hs578T and HBL100 cell lines have a high proportion (>90%) of CD44+/CD24−/low cells, and the CD44+/CD24−/low subpopulation obtained from these cell lines were capable of forming tumors in NOD/SCID mice and were more resistant to chemotherapy (Fillmore and Kuperwasser, 2008). In addition, these potential Claudin-low cell lines share gene expression profiles with the normal breast bipotent subpopulation of Raouf et al. (2008) (CD49f+/MUC1−CD133−(CD10/THY1)+) and the MaSC subpopulation of Lim et al. (2009) (CD49f high/EpCAM−). Moreover, we have shown that the SUM159PT Claudin-low cell line possesses a similar antigenic phenotype as the MaSC subpopulation of Lim et al. (2009) with positivity for CD49f and low to absent expression of EpCAM (Prat et al., 2010). Of note, EpCAM is currently being used as the antigen to isolate circulating tumor cells by the CellSearch method (Cristofanilli et al., 2004). Thus, if CSCs are EpCAM-negative, then it seems unlikely that circulating CSC in breast cancer patients will be detected by the CellSearch assay, which has already been shown to be the case for the majority of Claudin-low cell lines (Sieuwerts et al., 2009).

In general, breast cancer cell lines do not express the immune response and mesenchymal/ECM gene clusters observed in breast tumors (Figure 3A). This is likely due to the lack of contamination of non-epithelial cell types in *in vitro* epithelial cell cultures. However, Claudin-low cell lines are still highly enriched with genes involved in wound/inflammatory responses compared to the other cell lines (Figure 4A–B), concordant with the high expression of these same genes in Claudin-low tumors. Both data point to the interaction between CSCs and the cellular microenvironment as a key event in determining tumor growth and survival, which is supported by many recent preclinical studies (Kim et al., 2009; Charafe-Jauffret et al., 2009; Santisteban et al., 2009). For example, CD8 T-lymphoid cells can induce an EMT and a stem cell-like phenotype in epithelial cells from a murine breast cancer model (Santisteban et al., 2009), while highly metastatic cells of the MDA-MB231 cell line enhance tumor growth, angiogenesis and stromal recruitment by secreting interleukin 6 (IL-6) and interleukin 8 (IL-8) (Kim et al., 2009). Blockade of the IL-8 receptor CXCR1 using a CXCR1-specific blocking antibody or repertaxin (a small-molecule CXCR1 inhibitor), selectively depletes the CSC population of the Claudin-low cell line SUM159 (Ginestier et al., 2010). Thus, strategies to interfere with these inflammatory-related processes might be useful in the treatment of breast cancers in general, and for Claudin-low tumors specifically.

Given the low expression of Claudin proteins and E-cadherin in Claudin-low tumors, it might be possible to identify these tumors using a methodology like immunohistochemistry; however, we believe that classifications based upon the lack of marker(s) is a troublesome method for multiple reasons including 1) assay technical failure would yield false-negative
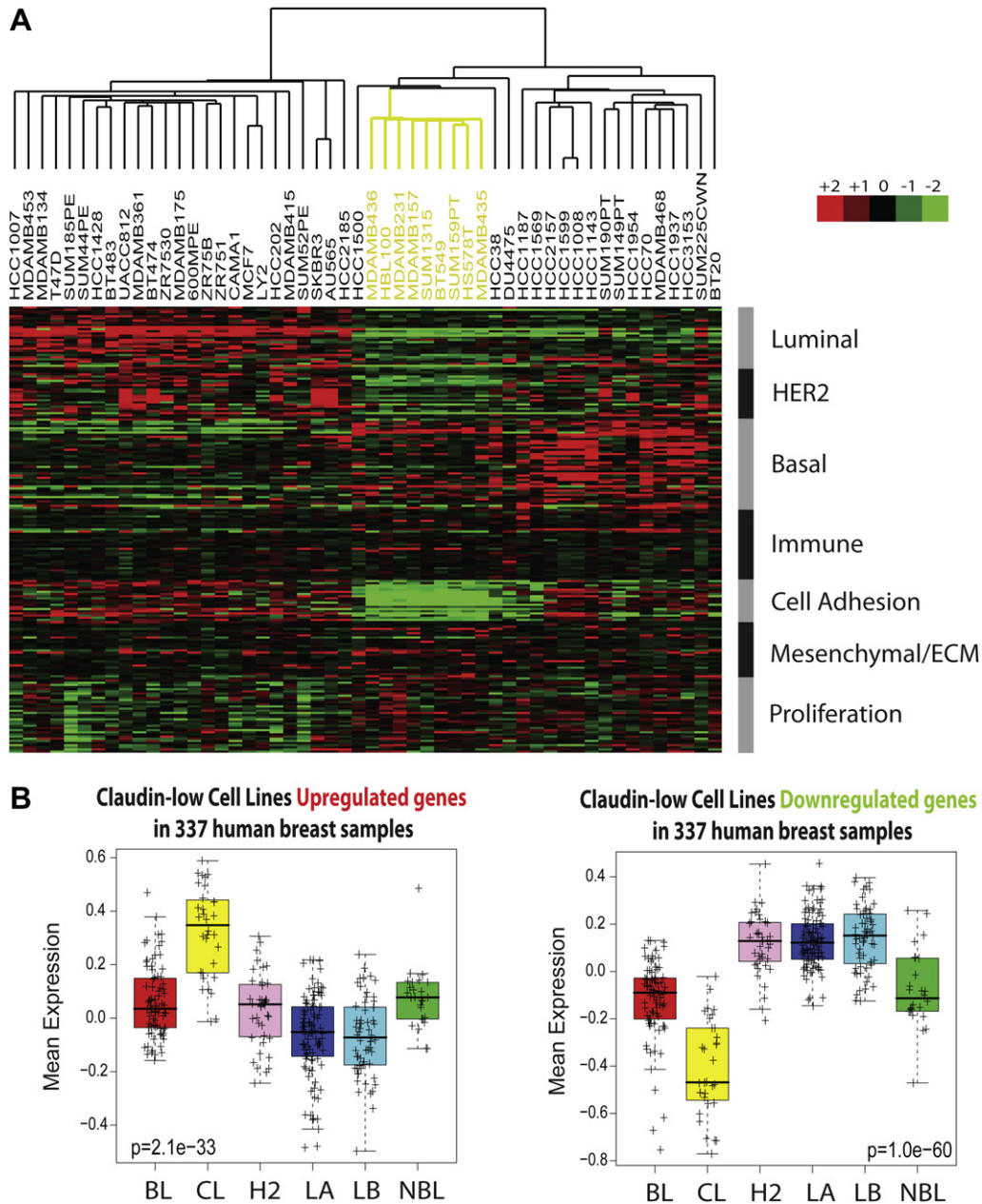
Figure 3 – Identification of the Claudin-low profile in breast cancer cell lines. (A) Intrinsic Gene clusters selected in Figure 1 are shown here using the cell line gene expression data set of Neve et al. (2006). The sample associated dendrogram has been derived by semi-unsupervised hierarchical clustering using the intrinsic list from Parker et al. (2009b) and the 51 cell lines of Neve et al. Claudin-low cell lines are shown in yellow. Each colored square represents the relative transcript abundance (in log2 space) with highest expression being red, average expression being black, and lowest expression being green. (B) Mean expression of the top highly expressed (*n* = 833) and lowly expressed (*n* = 642) genes in Claudin-low cell lines across 337 human breast tumor samples classified according to intrinsic subtype, including the Normal Breast-like group. Both gene lists were obtained by performing Significance Analysis Microarray (SAM) between Claudin-low breast cancer cell lines vs. the rest (FDR < 5%). BL, Basal-like; CL, Claudin-low; H2, HER2-enriched; LA, Luminal A; LB, Luminal B; NBL, Normal Breast-like. This figure has been modified from Prat et al. (2010).

results, 2) tumors derived from other tissues, or cell types, could yield false positives. For example, a poorly differentiated sarcoma of the breast might be called a Claudin-low breast tumor due to its location and lack of staining for Claudin 3. This point is particularly relevant given the wide-spread classification of breast tumors as "triple-negative" breast cancers. Therefore, to address this issue and using the cell line gene

expression data of Neve et al. (2006), we developed a ~800 gene Claudin-low centroid-based predictor (which contains genes whose high and low expression defines Claudin-low tumors) and applied it to our *in vivo* human breast tumor data set as a test/validation set. Using this cell line-based predictor, we were able to identify the Claudin-low human tumors samples with high sensitivity (87.5%) and specificity (97.0%) (Prat et al.,
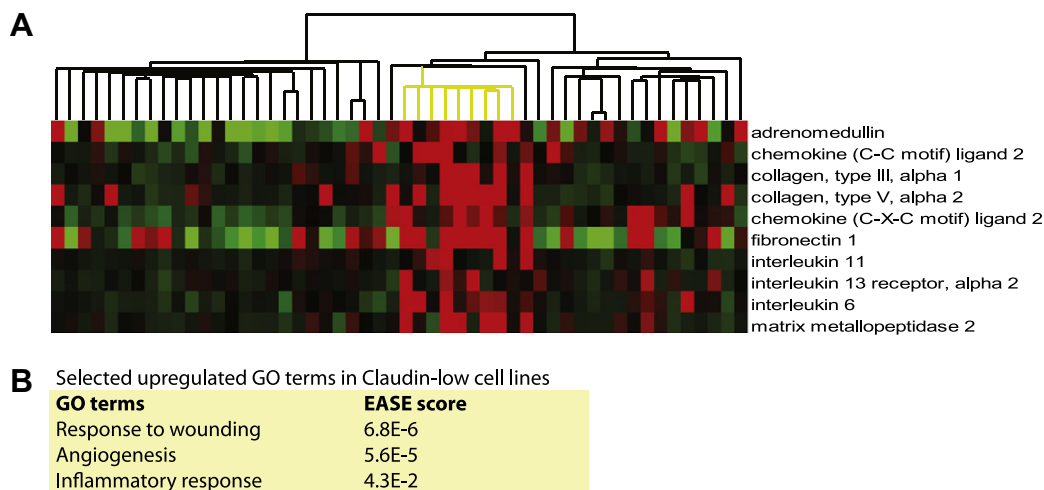
Figure 4 — Expression of inflammatory response/angiogenesis biological processes genes across 52 breast cancer cell lines. (A) Selected expression of genes involved in wound response, angiogenesis and/or inflammatory response. The sample/cell line associated dendrogram was derived by semi-unsupervised hierarchical clustering using the intrinsic list from Parker et al. (2009b). Claudin-low cell lines are shown in yellow color. (B) Selected highly expressed Gene Ontology (GO) terms in Claudin-low cell lines. The highly expressed gene list was obtained by SAM between Claudin-low breast cancer cell lines vs. rest (FDR < 5%). Biologic analysis of microarray data was performed with DAVID annotation tool (http://david.abcc.ncifcrf.gov/) (Dennis et al., 2003).

2010). We believe the 9-Cell Line Claudin-low predictor is currently the best method to identify these tumors and cell lines across microarray data sets; however, this predictor is sensitive to differences in data set diversity and across data set normalization methods because it is based upon relative gene expression levels. In addition, tumors with high tumor associated stromal content might also be identified as Claudin-low due to their similar gene expression patterns.

4. Clinical characteristics of the Claudin-low and the other intrinsic tumor subtypes

4.1. Clinical-pathological parameters and prognosis

The main clinical-pathological features of the molecular portraits of breast cancer, including the Claudin-low subtype, are shown in Figure 5A, which is based upon three independent microarray-based data sets (total $n = 748$). Overall, Claudin-low tumors are the least frequent subtype (prevalence ~12–14%) and are mostly high-grade and ER–/PR–/HER2– (i.e. triple-negative) tumors similar to the Basal-like subtype, which is concordant with the low expression of the luminal and HER2 intrinsic gene clusters observed in both tumor types. However, it is important to note that ~15–25% of Claudin-low tumors are hormonal receptor-positive (HR+) and ~10% of Basal-like tumors are also HR+.

In terms of patient outcomes, Claudin-low tumors are poor outcome tumors compared to luminal A tumors (Figure 5B). However, no differences in survival were observed between Claudin-low tumors and other poor prognosis subtypes (Luminal B, HER2-enriched and Basal-like), or even between Claudin-low tumors versus all other tumors combined. This

is in concordance with previous stem cell-like signatures that do not show prognostic ability as a whole, although subsets of genes within these signatures can predict outcome (Creighton et al., 2009; Shipitsin et al., 2007). At first glance, the invasiveness gene signature (IGS) reported by Liu et al. (2007) may seem an exception. However, the IGS was derived by comparing the gene–expression profile of CD44+CD24–/low tumorigenic breast cancer cells with normal breast epithelial cells (i.e. HMEC) and not versus differentiated (CD44– and/or CD24+) tumor cells as other studies have done (Creighton et al., 2009; Shipitsin et al., 2007). Therefore, the IGS likely distinguishes Luminal A tumors from the other poor prognostic subtypes, and it is possibly not focused on stem cell features but rather general poor prognosis tumor features.

Our data also show that the classical pathological markers used in the clinic for tumor classification (ER, PR and HER2) do not fully recapitulate the intrinsic subtypes (Figure 6). As previously shown by Parker et al., 2009b, this finding demonstrates that ER, PR and HER2 status alone, or in combination, are not accurate surrogates for true intrinsic subtype status. For example, in a combined data set of ~400 tumors/patients (UNC337 (Prat et al., 2010) and MDACC133 (Hess et al., 2006)) (Figure 6A), 49% of triple-negative tumors were Basal-like, 30% Claudin-low, 9% HER2-enriched, 6% Luminal B, 5% Luminal A and 1% Normal Breast-like; if the Claudin-low classification is ignored, then 72% of triple-negative tumors are Basal-like. Conversely, 6–29% (Sorlie et al., 2001; Nielsen et al., 2004) and 9–13% (Sorlie et al., 2001) of Basal-like tumors are ER+ or HER2+, respectively (Figure 6B). Thus the triple-negative surrogate for Basal-like makes both kinds of mistakes in that it includes samples that are not Basal-like and it fails to identify a significant number of Basal-like tumors (Figure 6A). Preliminary data suggest that Basal-like tumors that are not triple-negative behave as Basal-like tumors that
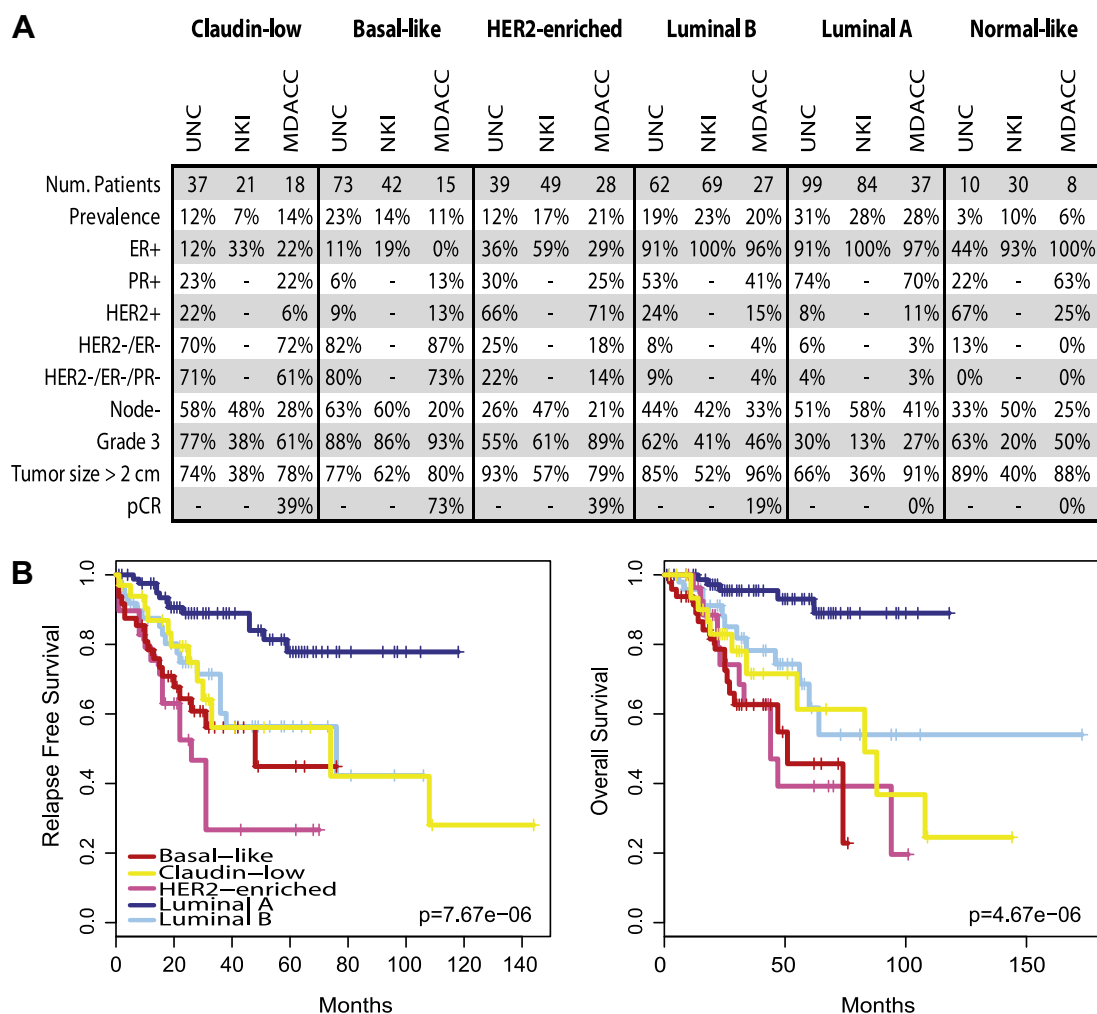
A	Claudin-low			Basal-like			HER2-enriched			Luminal B			Luminal A			Normal-like		
	UNC	NKI	MDACC	UNC	NKI	MDACC	UNC	NKI	MDACC	UNC	NKI	MDACC	UNC	NKI	MDACC	UNC	NKI	MDACC
Num. Patients	37	21	18	73	42	15	39	49	28	62	69	27	99	84	37	10	30	8
Prevalence	12%	7%	14%	23%	14%	11%	12%	17%	21%	19%	23%	20%	31%	28%	28%	3%	10%	6%
ER+	12%	33%	22%	11%	19%	0%	36%	59%	29%	91%	100%	96%	91%	100%	97%	44%	93%	100%
PR+	23%	-	22%	6%	-	13%	30%	-	25%	53%	-	41%	74%	-	70%	22%	-	63%
HER2+	22%	-	6%	9%	-	13%	66%	-	71%	24%	-	15%	8%	-	11%	67%	-	25%
HER2-/ER-	70%	-	72%	82%	-	87%	25%	-	18%	8%	-	4%	6%	-	3%	13%	-	0%
HER2-/ER-/PR-	71%	-	61%	80%	-	73%	22%	-	14%	9%	-	4%	4%	-	3%	0%	-	0%
Node-	58%	48%	28%	63%	60%	20%	26%	47%	21%	44%	42%	33%	51%	58%	41%	33%	50%	25%
Grade 3	77%	38%	61%	88%	86%	93%	55%	61%	89%	62%	41%	46%	30%	13%	27%	63%	20%	50%
Tumor size > 2 cm	74%	38%	78%	77%	62%	80%	93%	57%	79%	85%	52%	96%	66%	36%	91%	89%	40%	88%
pCR	-	-	39%	-	-	73%	-	-	39%	-	-	19%	-	-	0%	-	-	0%

Figure 5 − Clinical-pathological characteristics of the current intrinsic subtypes of breast cancer. (A) Table summarizing the percentages of the different pathological variables across three microarray data sets with clinical information (UNC337, NKI295 (van 't Veer et al., 2002) and MDACC133). (B) Kaplan−Meier relapse-free survival and overall survival curves using the UNC337 data set with Normal Breast-like samples excluded. This figure has been modified from Prat et al. (2010).

are, which may be clinically important if therapies are found that target the unique biology of Basal-like cancers.

Previous studies (including our own) have tried to define Basal-like carcinomas based on immunohistochemical (IHC) surrogate profiles. For example, EGFR and keratins 5/6 (CK5/6) (Figure 2D−E) have been proposed as positive IHC markers on top of the ER-PR-HER2- definition (the "five-marker method", also known as the Core Basal group). This definition has previously been shown to identify Basal-like tumors versus microarray-based classifications with 76% sensitivity and 100% specificity (Nielsen et al., 2004). Furthermore, in a series of 4046 breast tumors (Cheang et al., 2008), 17% (639 of 3744) were defined as the triple-negative, whereas 9.0% were Basal-like by the five-marker Core Basal definition. Interestingly, when the triple-negative group was segregated into Core Basal and the '5 Negative Profile' (5NP), the Core Basal group showed a significantly worse outcome compared to the 5NP group. Thus, although two distinct groups within triple-negative tumors seem to be identified, further paired microarray-IHC studies should determine whether the 5NP group resembles or enriches for the Claudin-low subtype. However, as shown in Figure 6B, up to ∼30% of Claudin-low tumors do not fall into the ER−/HER2- clinical category.

Many efforts are being devoted to try to identify those patients with good outcome, and as shown in Figure 5B, patients with a low-risk of relapse are found almost exclusively in the Luminal A subtype (Parker et al., 2009b; Fan et al., 2006). Thus, there is a need to find biomarkers that can distinguish Luminal A from Luminal B tumors, both of which are mainly HR+ (Figure 2F−G). A major biological difference between luminal A and B is the proliferation signature, which has higher expression in luminal B tumors than in luminal A tumors (Cheang et al., 2009; Nielsen et al., 2010); histological grade also mirrors this proliferation difference (Figure 2F−G). Indeed, proliferation is a main "driver" of the majority of genomic predictors designed to separate ER-positive lymph node-negative tumors into prognostic subgroups (Sotiriou et al., 2006). For example, the expression of proliferation related
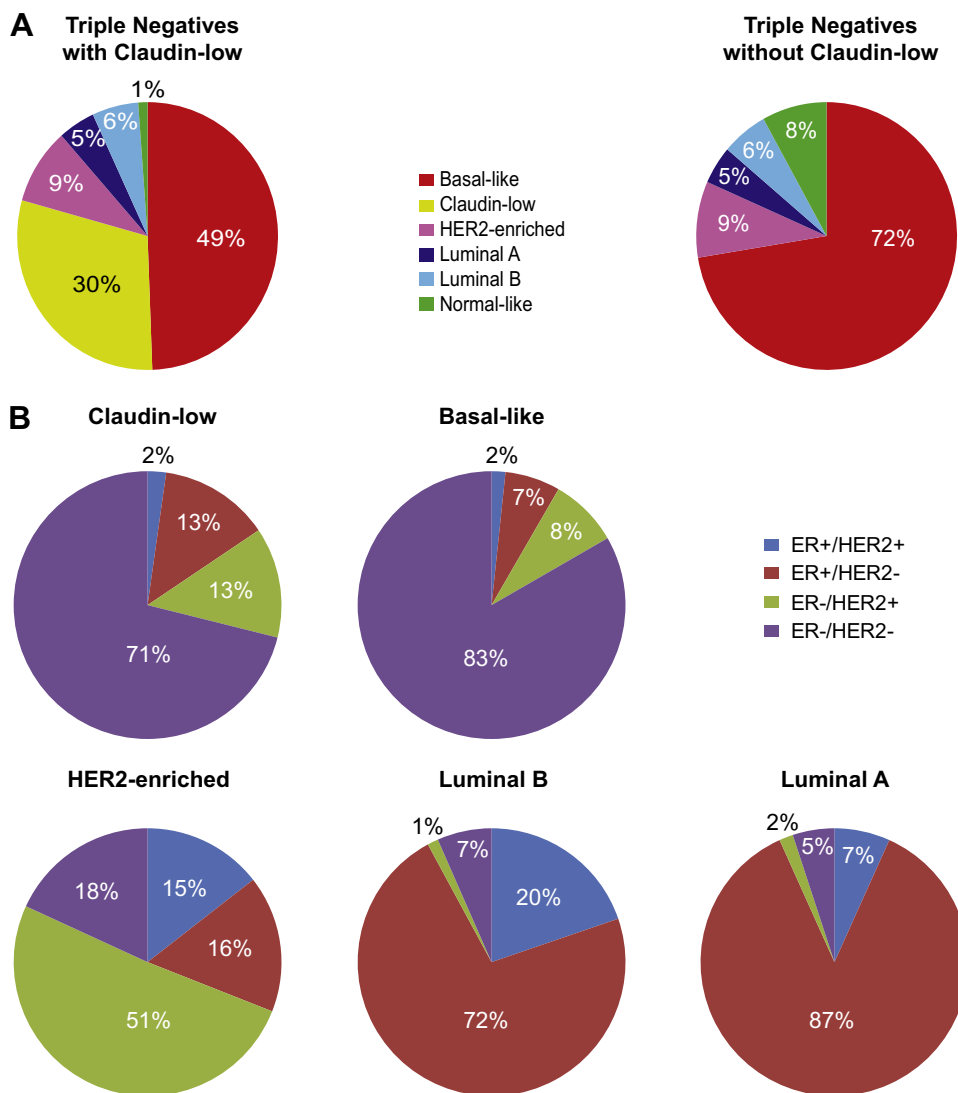
Figure 6 − Distribution of clinical-pathological categories relative to the intrinsic subtypes of breast cancer. (A) Intrinsic subtype distribution within the triple-negative tumor category shown with and without Claudin-low tumors. (B) Distribution of ER+/HER2+, ER−/HER2+, ER−/HER2− clinical groups in the Claudin-low, Basal-like, HER2-enriched, Luminal B, and Luminal A within each subtype.

genes, such as MKI67 (encoding Ki-67) and Cyclin B1, are the most heavily weighted component in calculating the recurrence score derived from the OncoTypeDX assay (Paik et al., 2004). Thus, these and other similar prognostic predictors classify virtually all Luminal B tumors, as well as all the Basal-like and HER2-enriched tumors, as high risk of recurrence (Fan et al., 2006). It is important to note here that recent studies have questioned the reproducibility, and thus the relevance of the Luminal B distinction, which is likely due to the large amount of heterogeneity seen within luminal cancers. For example in Parker et al. (2009b), at least 5 subgroups of Luminal cancers were seen (see Figure A1). Since there appears to be only one good outcome luminal subtype, but multiple types of poor outcome luminal tumors, proliferation scores and other means of identifying samples that have deviated away from the prototypical Luminal A profile are what the current ER+ prognosticators are doing (like OncotypeDX and Mammaprint). It is also because of this heterogeneity within

Luminal tumors that the Risk or Relapse (ROR) score was developed in Parker et al. (2009b), which should be less sensitive to changing distributions of poor outcome subtypes of disease when compared versus a nearest centroid predictor.

The protein expression of Ki-67 has been studied as a potential IHC marker that could distinguish Luminal B from Luminal A subtypes in HR+ breast tumors. In Cheang et al. (2009), 357 breast tumors were profiled and tumor subtypes were assigned using the 50-gene qRT-PCR 'PAM50' subtype predictor that we have recently validated (Parker et al., 2009b). By linking the available immunohistochemical data with the expression profile assignments, the authors identified 84 and 60 HR+/HER2− tumors as Luminal A and B, respectively. Thus, the Luminal A subtype was defined as being HR+/HER2− and low for Ki-67, and the Luminal B subtype as being HR+/HER2− and high for Ki-67 or HR+/HER2+. Further validation of this surrogate IHC panel in an independent population-based cohort of 4046 tumors demonstrated the prognostic value of this Luminal

B IHC definition within homogeneously treated patient subsets. However, we must keep in mind that although the HR+/HER2−/Ki67-high/low IHC panel will distinguish the majority of Luminal B from A tumors, this definition does not identify all the tumors within the Luminal B expression-defined subtype since up to 20% and 7% of Luminal B tumors are clinically ER+/HER2+ and ER−/HER2−, respectively (Figure 6B).

Finally, the HER2-enriched subtype consists of samples that are mostly clinically HER2+ (Figure 2H), highly proliferative, lack expression of the basal cluster, and show low expression of the luminal cluster compared to Luminal A and B tumors. As seen with the others subtypes, IHC markers (i.e. ER−/HER2+) are not an accurate surrogate for this particular intrinsic subtype, since only ∼50% of HER2-enriched tumors are ER−/HER2+. As shown in Figure 6B, 49% of HER2-enriched tumors are divided into the following clinical categories: ER+/HER2+ (15%), ER+/HER2− (16%) and ER−/HER2− (18%). It is important to note that although ∼30% of HER2-enriched tumors are clinically HER2- (hence the subtype name of HER2-*enriched*), these tumors might be driven by a similar functional event such as the HER2 mutation or mutation of some downstream pathway component that phenocopies HER2 amplification.

4.2. Metaplastic and medullary breast carcinomas, BRCA1 dysfunction, and the Claudin-low/stem cell-like profile

The majority of Claudin-low tumors are invasive ductal carcinomas not otherwise specified (IDC NOS), which is the most frequent histological diagnosis in breast cancer (WHO Classification of Tumors, 2003). However, metaplastic and medullary carcinomas have also been linked with the Claudin-low profile (Prat et al., 2010; Hennessy et al., 2009). These two special histological types represent less than 5−7% of all breast cancer diagnoses (WHO Classification of Tumours, 2003), and generally are poorly differentiated triple-negative tumors. However, while metaplastic carcinomas are associated with poor prognosis and treatment resistance (Hennessy et al., 2005; Al Sayed et al., 2006), medullary carcinomas tend to show good outcomes despite their aggressive pathological features (Vu-Nishino et al., 2005).

In Hennessy et al. (2009) the expression profiles of 12 metaplastic carcinomas (MBC) were compared with 184 breast tumors (mainly IDC NOS) and 9 normal breast samples. MBC were somewhat heterogeneous in this analysis with 2 MBC clustering with the Claudin-low tumors, 2 with the Basal-like tumors, and 6 formed a potential novel subgroup of tumors intermediate between Basal-like and Claudin-low tumors. However, the majority of these metaplastic tumors ($n = 7/12$) were further identified as Claudin-low by the 9-Cell Line Claudin-low predictor (Prat et al., 2010). In addition, metaplastic tumors as a group were found to be enriched with a CD44+/CD24^{low/−} stem cell-like gene signature similar to Claudin-low tumors (Hennessy et al., 2009), suggesting that metaplastic carcinomas and Claudin-low tumors possess similar transcriptional features that are enriched in purified breast TIC fractions.

In Prat et al. (2010), 5 of 21 (24%) Claudin-low tumors showed medullary-like features such as pushing margins and brisk tumor lymphocytic infiltration (Figure 2I). As

defined by Ridolfi et al. (1977), medullary carcinomas are divided into 2 categories: typical and atypical medullary carcinomas. Typical medullary carcinomas display at least 75% syncytial architecture, marked anisonucleosis, a well-defined margin, diffuse lymphoplasmocytic infiltrate, and absence of tubular differentiation and/or an intraductal component. Atypical medullary carcinomas also have the syncytial architecture and at least two or three of the above criteria. By strict definition, the Claudin-low tumors with medullary-like features identified in Prat et al. (2010) did not meet Ridolfi's criteria to be called either typical or atypical medullary. However, further microarray analyses demonstrated that medullary carcinomas as a group share similar MaSC gene expression profiles as do Claudin-low tumors, which is concordant with another report (Honeth et al., 2008) that showed that 8/8 medullary carcinomas were positive for the CD44+/CD24− phenotype by IHC staining. The link between medullary carcinomas (as well as metaplastic carcinomas) with the Claudin-low gene expression profile was further observed in a comprehensive data set of 113 tumors from 11 special histological types of breast cancer, including 10 medullary and 20 metaplastic carcinomas (Weigelt et al., 2008). In this data set, 20% and 40% of medullary and metaplastic tumors previously called Basal-like were now identified as Claudin-low by the 9-Cell Line Claudin-low predictor (Prat et al., 2010). Interestingly, the Claudin-low breast cancer cell lines MDA-MB157 and Hs578T were derived from medullary (Young et al., 1974) and metaplastic (Hackett et al., 1977) carcinomas, respectively.

The fact that a subset of medullary and metaplastic carcinomas share Claudin-low gene expression profiles indicates that these tumors might share a common cell of origin, and/or similar initiating genetic event(s). One of these oncogenic alterations may involve the BRCA1 pathway. Both metaplastic and medullary carcinomas have been shown to have a ∼60% incidence of methylation of BRCA1 (Turner et al., 2006; Esteller et al., 2000). 13% of breast tumors from BRCA1 mutation carriers have pure medullary histology (Eisinger et al., 1998), while 60% show medullary-like features (Lakhani et al., 1998), especially pushing margins and lymphoid infiltration. Metaplastic tumors also have been recently documented in this particular patient subpopulation (Suspitsin et al., 2009). It is interesting to note that 2/4 BRCA1-mutated breast cancer cell lines are Claudin-low (MDA-MB436 and SUM1315), while the other 2 are Basal-like (SUM149PT and HCC1937) (Elstrodt et al., 2006).

To further explore the association between BRCA1-mutated breast cancer and the Claudin-low subtype, we applied the Claudin-low predictor to the NKI ($n = 337$) microarray data set (van 't Veer et al., 2002; van de Vijver et al., 2002), which includes 18 BRCA1-mutated breast tumors. Of the 18 BRCA1 mutant tumors, we identified 12 as Basal-like (67%), 4 as Claudin-low (22%), 1 as HER2-enriched (5.5%), and 1 as Normal Breast-like (Prat and Perou, unpublished observation). This finding is concordant with a 20% incidence of Claudin-low/mesenchymal tumors observed in the Brca1Co/Co; TgMMTV-Cre; p53+/− breast cancer mouse model (Herschkowitz et al., 2007). Thus, although BRCA1 mutations are most frequent in Basal-like tumors, they may also occur within the Claudin-low subtype and further studies should

determine differences in prognosis and treatment response between BRCA1-mutated Claudin-low and Basal-like tumors.

4.3. Intrinsic subtyping in the adjuvant and neoadjuvant setting

Current knowledge of the biology of breast cancer has provided the basis of the various successful adjuvant and neoadjuvant treatment strategies: endocrine therapy for HR+ disease (with or without chemotherapy), anti-HER2 therapies such as trastuzumab in combination or sequentially after chemotherapy for HER2+ disease, and chemotherapy for patients with triple-negative disease (Podo et al.). However, the biological diversity displayed by the breast cancer intrinsic subtypes indicate that further sub-classification of patients into different treatment groups should be considered.

Two studies have directly evaluated the response to neoadjuvant chemotherapy of the intrinsic subtypes as determined by gene expression (Rouzier et al., 2005a; Parker et al., 2009a). Rouzier et al. (2005a) evaluated 82 primary breast tumors treated with 12 weeks of paclitaxel (T) followed by 4 cycles of 5-flourouracil, doxorubicin, and cyclophosphamide (FAC). Surgery was performed after 24 weeks of neoadjuvant therapy and patients were evaluated for pathological complete response (pCR). Among 22 patients with Basal-like tumors and the 20 patients with HER2-enriched tumors, the pCR rates were both 45%, whereas only 7% of Luminal A/B tumors achieved a pCR. More recently, Parker et al. (2009b) evaluated the ability of the molecular subtypes to predict pCR to anthracycline/taxane-based chemotherapy using a combined cohort of 357 patients from three different neoadjuvant studies (Parker et al., 2009a). Among the subtypes, Basal-like and HER2-enriched tumors showed the highest response rate with 43% and 36% pCR rates, respectively, whereas Luminal A and B tumors showed 7% and 17% pCR rates. Multivariable logistic regression indicated that intrinsic subtype was an independent predictor of pCR and ER status was no longer significant when subtype was included in the model. These studies highlight the higher chemo-sensitivity of Basal-like and HER2-enriched subtypes (largely ER-negative) and the chemo-insensitivity of the Luminal subtypes (largely ER-positive), which explains why ER status is such a strong predictor of pCR among the various clinical variables (Carey et al., 2007; Rouzier et al., 2005b). The relative insensitivity of Luminal/ER+ tumors may be due to an intact ER-cMYB-HEP27-MDM2-TP53 response cascade, which may allow these tumors to go into a TP53 and p21 mediated cell cycle arrest in response to chemotherapy treatment (Deisenroth et al., 2010). These studies illustrate the need to account for varying subtype proportions when comparing pCR statistics across clinical trials.

The relationship between subtype and chemotherapy response has also been evaluated using IHC surrogates for the molecular subtypes. In Carey et al. (2007), 107 patients were treated with neoadjuvant AC for 4 cycles and followed for a median of 39 months. As expected, pCR to chemotherapy was significantly better among triple-negative (27%) and HER2+/ER− (36%) tumors versus ER+ tumors (7%). However, despite the lower rates of response to therapy, disease-free survival was still better for patients with ER+ tumors due to

higher rates of relapse in triple-negative and HER2+/ER− patients with residual disease. Known as the "triple-negative paradox", this hypothesis was further tested retrospectively by Liedtke et al. (2008) by comparing response to neoadjuvant chemotherapy and survival using 1118 patients with triple-negative and non-triple-negative breast cancer. In this study, patients with triple-negative disease had significantly higher pCR rates when compared with non-triple-negative disease (22% vs. 11%), but showed decreased 3-year progression-free survival and overall survival rates. More importantly, both triple-negative and non-triple-negative patients had similar survival if pCR was achieved, thus some triple-negative patients can have good long term survival outcomes. In contrast, patients with residual disease had worse overall survival if they had triple-negative disease compared with non-triple-negative disease. Thus, the pCR surrogate marker after chemotherapy seems appropriate for Basal-like and Claudin-low subtypes, which represent ∼80% of all triple-negative tumors.

The endocrine treatment sensitivity of Luminal A versus Luminal B subtypes has not been specifically studied until recently (Cheang et al., 2009; Nielsen et al., 2010). In Cheang et al. (2009), among 976 tumors from patients treated with tamoxifen as their only adjuvant systemic therapy, the authors identified 584 as luminal A, 303 as luminal B, and 89 as luminal/HER2+ (defined as ER+ or PR+ and HER2+) by using a surrogate immunohistochemical panel whose performance was previously trained using the PAM50 predictor (see above). The 10-year relapse-free survival was 70% survival for patients with luminal A (HR+/HER2−/Ki67low) tumors, and dropped to 53% for patients with luminal B (HR+/HER2−/Ki67high) tumors, and 51% for patients with luminal/HER2+ tumors. A more recent analysis using the more precise qRT-PCR PAM50 assay and tumors from the same University of British Columbia cohort of ER-positive tumors treated with tamoxifen-only confirmed these survival results, and was able to identify a set of very good outcome patients whose 20 year survival probability was ∼95% (Nielsen et al., 2010); thus, high proliferative Luminal B tumors have a worse prognosis despite treatment with tamoxifen, while Luminal A/Risk of Relapse (ROR)-low tumors show favorable relapse-free and disease specific survival outcomes after treatment with tamoxifen alone in node-negative patients. Furthermore, treatment with aromatase inhibitors might not change the overall hormone-resistance of Luminal B tumors. In the TransATAC study, high recurrence scores in the primary tumor as determined by the OncotypeDX assay were independently associated with higher risk of relapse in 1308 HR+ node-negative and node-positive patients treated with anastrozole (or tamoxifen) (Dowsett et al., 2008). As previously discussed, HR+ tumors with a high recurrence score are mostly Luminal B cancers (Fan et al., 2006). In addition, features of the Luminal B subtype such as high Ki-67 and low ER status in postsurgical samples, were independently associated with relapse-free survival after treatment with neoadjuvant letrozole or tamoxifen (P024 trial) (Ellis et al., 2008). Luminal B tumors are not only relatively chemo-insensitive tumors, but they are also poor prognostic and relatively hormone-resistant tumors. Clinical trials focusing in this particular luminal subtype are needed and several are in the planning stage.

The known mechanisms of resistance to hormonal therapy may help decipher the biology of Luminal B tumors (Loi et al., 2008). Among them, ligand-independent activation of ER by the epidermal growth factor receptor (HER) family has been extensively studied (Osborne and Schiff, 2005). In particular, HER2 overexpression and/or amplification (HER2+) confers increased resistance to endocrine treatment in preclinical models (Benz et al., 1992; Pietras et al., 1995) and in ER-positive breast cancer patients treated with tamoxifen (Houston et al., 1999; Lipton et al., 2003) or AIs (Lipton et al., 2003; Ellis et al., 2006). The HER family of receptors includes EGFR (also known as HER1), HER2, HER3, and HER4. HER2 is a ligand-less receptor that forms homodimers and heterodimers with the other members of the HER family, resulting in activation of signal transduction pathways that increase proliferation such as the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3'-kinase (PI3K)/mTOR. A variety of kinases from both pathways can phosphorylate specific sites of the ER, leading to ligand-independent ER activation. Conversely, ER itself can activate the growth factor receptor pathway either through genomic and/or nongenomic signaling (Osborne and Schiff, 2005). Thus, in HR+/HER2+ breast tumors, which represent 20% of Luminal B tumors, a vicious cycle might be established between ER mechanisms of action and HER2 leading to enhanced cell proliferation and cell survival. Two clinical trials have tested this hypothesis and have demonstrated that a combined endocrine and anti-HER2 approach significantly enhances progression-free survival and clinical benefit rates in patients with HR+/HER2+ metastatic breast cancer (Johnston et al., 2009; Kaufman et al., 2006).

In HR+/HER2-/high proliferative tumors, which represent ~72% of tumors in the Luminal B subtype, the HER-pathway might also be active in a different manner than HER2 amplification. For example, overexpression of HER2 in luminal breast cancer cell lines is not required for HER-signaling if HER ligands are available (Agus et al., 2002; Menendez et al., 2006). In addition, gene expression profiling of luminal MCF-7 cells treated with HER3 ligand heregulin (HRG) identified a similar expression profile as the Luminal B subtype in patients (Loi et al., 2009). Thus, activation of the HER-pathway and/or similar downstream functional pathways such as the PI3K/mTOR pathway could explain the shared biology between ER+/HER2-/high proliferative tumors and ER+/HER2+ tumors, both of which are contained within the Luminal B subtype.

A successful targeted treatment strategy has been the development of anti-HER2 therapies such as trastuzumab, an anti-HER2 monoclonal antibody. In patients with HER2+ tumors, administration of trastuzumab in the adjuvant setting in combination with chemotherapy results in an improvement in recurrence-free survival as well as overall survival (Piccart-Gebhart et al., 2005; Romond et al., 2005). Despite this success, many HER2+ patients do not benefit from trastuzumab, which has led to many efforts to identify additional biomarkers of response/benefit to anti-HER2 therapies. Once again, the molecular subtypes might help better understand the biology of this responsiveness. Looking at HER2-positivity across subtypes reveals that although the majority of HER2+ tumors have a HER2-enriched gene expression profile, all the other intrinsic subtypes have HER2+ tumors within them, indicating that the HER2+ clinical category is biologically heterogeneous. Thus, further clinical trials should try to answer these two challenging questions: (1) does the HER2-enriched subtype "enrich" for HER2+ patients that have a higher response/benefit to anti-HER2 therapies than HER2+ and non-HER2-enriched tumors? (2) do patients with clinically HER2- tumors within the HER2-enriched subtype benefit from anti-HER2 therapies? This question will be interesting to address given recent suggestions that some HER2-patients may gain a trastuzumab benefit (Paik et al., 2008), however, this can only be addressed through the analysis of trials where all patients were given trastuzumab, which are few.

Another challenge will be to identify effective targets for the triple-negative subpopulation, which is basically composed of Basal-like and Claudin-low tumors. The list of molecular targets that are being evaluated here is constantly growing and includes: poly(adenosine diphosphate [ADP]−ribose) polymerase 1 (PARP1), vascular endothelial growth factor (VEGF, see Figure 1C), HER1, MAPK, PI3K/mTOR, and the stem cell pathways NOTCH and Hedgehog. One of the promising therapies being tested on this subgroup of tumors are inhibitors of PARP1, a key player in the repair of DNA single-stand breaks. As noted earlier, Basal-like and potentially Claudin-low tumors, are commonly seen in BRCA1 mutation carriers (Foulkes et al., 2003), and defects in the BRCA1 pathway may also be involved in sporadic tumors of both subtypes. Dysfunctional BRCA1 results in deficient DNA-repair by homologous recombination, which causes genetic aberrations that drive carcinogenesis. The inhibition of PARP1 in this BRCA1 dysfunctional context leads to the accumulation of collapsed replication forks, DNA double-strand breaks, and cell death (Rottenberg et al., 2008). Defect or inhibition of these genes individually is tolerable, yet in combination is lethal, a concept called synthetic lethality. A phase 1 clinical trial evaluated olaparib (AZD2281) as a single agent in a study patient population composed of hereditary BRCA1 and BRCA2 mutated cancers observed an anti-tumor activity of ~60%. A subsequent randomized phase 2 clinical trial evaluated another PARP1 inhibitor, BSI-201, in combination with gemcitabine/carboplatin in 123 subjects with sporadic triple-negative metastatic breast cancer (O'Shaughnessy et al., 2009). Preliminary analysis demonstrated that the addition of BSI-201 to standard chemotherapy doubled the progression-free survival compared to chemotherapy alone. Intriguingly, PARP1 and other DNA-repair pathway-related genes such as CHEK1 are typically found highly expressed in Basal-like cancers, suggesting they may be under a constant state of DNA-repair, which may not be seen in Claudin-low tumors (Figure 1B). Further studies should determine if inhibition of PARP1 is a Basal-like specific therapy, or alternatively, a therapy that may increase the chemo-sensitivity in any breast cancer that is homologous recombination DNA-repair deficient, regardless of subtype.

5.   The CSC hypothesis, treatment resistance, and the intrinsic subtypes

According to the cancer stem cell (CSC) hypothesis, a cancer arises either from transformation of a normal stem/progenitor cell with the inherent capacity to self-renew and to

differentiate into the various cell types that form the bulk of the tumor, or alternatively, arises from a differentiated cancer cell that acquires the ability to self-renew (Rosen and Jordan, 2009; Jordan, 2009). Either way, the CSC hypothesis suggests that cells with CSC features exist and represent a minor subpopulation within a tumor, but that they are the driver of the entire tumor phenotype.

The CSC hypothesis has important implications for breast cancer treatment strategies since breast CSCs are more resistant to both radiation and chemotherapy (Creighton et al., 2009; Gupta et al., 2009; Li et al., 2008; Diehn et al., 2009; McDermott and Wicha, 2010). In Li et al. (2008), paired breast cancer biopsies were obtained from 52 breast cancer patients before and after 12 weeks of treatment with either neoadjuvant chemotherapy or after 6 weeks of treatment with the EGFR/HER2 inhibitor lapatinib in HER2+ patients. The authors observed that chemotherapy treatment increased the percentage of CD44+/CD24−/low cells and the mammosphere formation efficiency (MSFE) regardless of the ER/PR/HER2 status of the tumor, while in the lapatinib group this increase was not observed suggesting that anti-HER2 therapies in HER2+ disease specifically target cancer stem cells. More recently, Creighton et al. (2009) derived a gene signature from CD44+/CD24−/low and cancer mammospheres (MMS) cells (both isolated from primary human breast cancers) by comparing these cells with the CD44−/CD24+ cell fraction. To examine the signature's clinical and therapeutic significance, the authors evaluated gene expression profiles of breast tumors before and after chemo- and hormone therapy. The CD44+/CD24−/low−/MMS signature was more pronounced in tumor tissue remaining after either endocrine therapy (letrozole) or chemotherapy (docetaxel), consistent with the selective survival of CSCs after treatment. In addition, the authors observed an increased expression of mesenchymal markers such as vimentin in cytokeratin-positive epithelial cells in the post-letrozole treated samples (Gupta et al., 2009).

The CD44+/CD24−/low/MMS gene expression profile of Creighton et al. (2009) has also been compared to the intrinsic subtypes of breast cancer, including the Claudin-low subtype. Of note, only the Claudin-low group showed a clear enrichment for the CD44+/CD24−/low/MMS signature, further suggesting that these tumors might be enriched with CSCs/TICs. To further explore the potential association between Claudin-low tumors and treatment resistance, we evaluated the potential chemo-sensitivity of Claudin-low tumors to neoadjuvant anthracycline/taxane-based chemotherapy using a cancer patient data set of 133 pre-treated samples (Prat et al., 2010). In this data set, Claudin-low tumors showed a trend towards a lower pathological complete response (pCR) rate after anthracycline/taxane-based chemotherapy compared to Basal-like tumors (39% vs. 73% pCR rates, Figure 5A), but their pCR rate was significantly higher than Luminal A (0%) or B (19%) tumors. These findings provided the first evidence that Claudin-low tumors show some chemotherapy insensitivity; however, their pCR rate was still significantly high, suggesting that they are not entirely therapy resistant. Further studies are needed to better characterize the treatment sensitivity of this tumor subtype, with Claudin-low cell lines and transgenic mouse models offering a good opportunity to tackle this issue preclinically.

6.   Developmental origins of the intrinsic subtypes

The first evidence that the breast cancer intrinsic subtypes might resemble different developmental cells types of the normal breast came from a recent report by Lim et al. (2009) where they functionally characterized and expression profiled different subpopulations of normal mammary epithelial cells using FAC sorting with two cell surface markers: EpCAM and CD49f. More precisely, the authors isolated highly purified subpopulations of normal human MaSC/bipotent cells (CD49f hi/EpCAM−), committed luminal progenitor cells (CD49f+/EpCAM+), and mature ER+/luminal cells (CD49f−/EpCAM+). Importantly, the authors observed that the luminal progenitor signature was very similar to the Basal-like breast tumor signature. The authors also demonstrated that BRCA1-mutated pre-neoplastic breast tissues, which are known to have a lifetime risk of developing Basal-like breast cancer of >80%, showed an aberrant expansion of this luminal progenitor subpopulation. These results, together with recent data coming from a genetically engineered mouse models study that deleted BRCA1 in the mouse mammary epithelial luminal progenitors (Molyneux et al., 2010), suggest that the potential cell of origin of sporadic and BRCA1-mutated Basal-like breast cancers could be the luminal progenitor (which shows the Basal-like tumor expression profile); however, other plausible hypotheses exist and are discussed below.

Similar to these Lim et al. (2009) observations, we hypothesized that a luminal epithelial differentiation program from a MaSC→Luminal progenitor→Mature luminal might exist (Figure 7A). To genomically evaluate this hypothesis, we used Lim et al.'s FACS data to derive a continuous "differentiation predictor model" for the luminal pathway (Prat et al., 2010). Using this differentiation score predictor and genomic data of human breast tumors, we observed that the majority of invasive breast tumors can be placed along the normal mammary luminal epithelial differentiation hierarchy starting with the Claudin-low subtype being closest to the MaSC, followed by the Basal-like, then the HER2-enriched, and then both Luminal tumors subtypes being closest to the mature luminal cell (Prat et al., 2010). As shown in Figure 7A, this simplified mammary luminal epithelial differentiation axis goes from a mesenchymal state, through a basal-like state (that has characteristics of mesenchyme, basal and luminal cells) to a mature luminal state. We note that this differentiation hierarchy does not take into account the myoepithelial cell lineage development, which based upon the data of Lim et al. (2009) suggests that the myoepithelial linage breaks off from the luminal lineage before the Basal-like/luminal progenitor state (Figure 7A). Much less is known about myoepithelial cell development and where and when bi-potency is lost (Visvader, 2009), however, learning more about this lineage will likely provide important information about some of the rare breast cancer histological subtypes that show myoepithelial/basal-like differentiation including medullary and adenocystic carcinomas.

These luminal developmental lineage data could support the hypothesis that each 'intrinsic' subtype reflects a unique mammary epithelial cell state along the differentiation hierarchy, and that each transformed cell (i.e. cell of origin) could
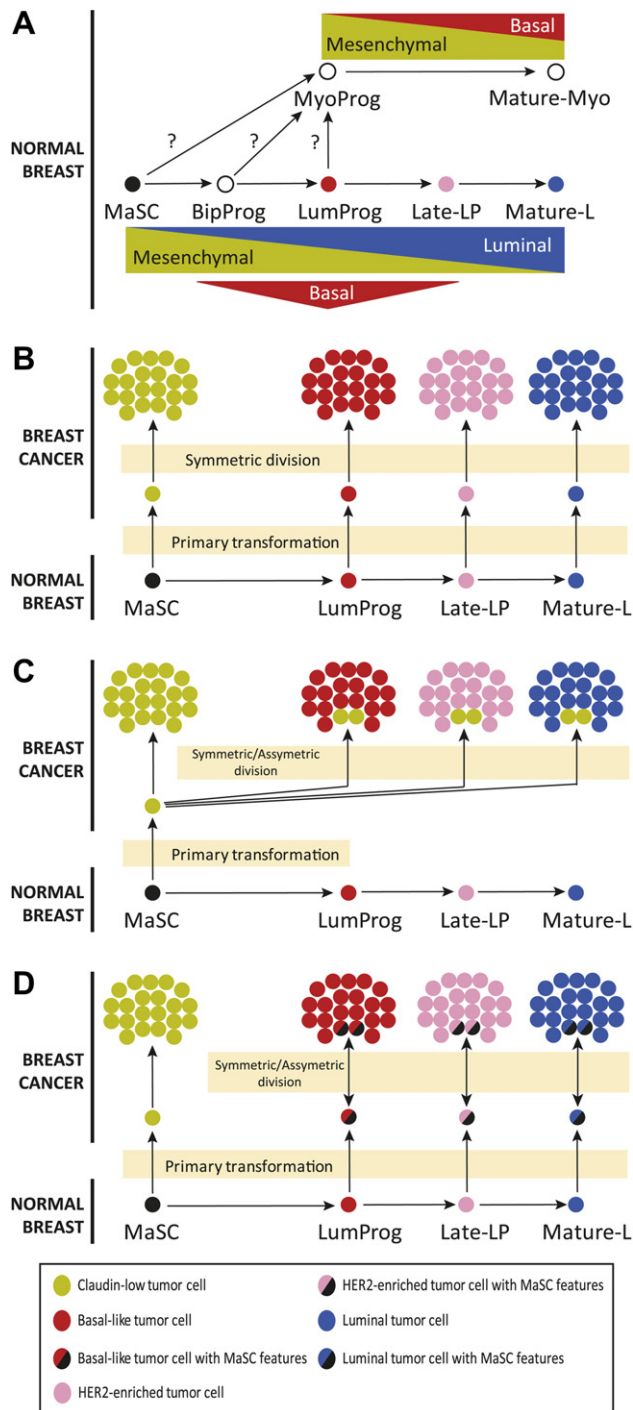
Figure 7 — Possible developmental origins of the intrinsic breast cancer subtypes. (A) Normal mammary luminal and myoepithelial differentiation hierarchies with approximate genomic expression patterns of mesenchymal, basal, and luminal profiles highlighted. (B) Hypothesis 1: each molecular subtype originates in a normal epithelial cell along the luminal differentiation hierarchy. Aberrant symmetric division expands each subpopulation of cells to form the bulk of the tumor, which is composed entirely of TICs. (C) Hypothesis 2: the MaSC subpopulation is the cell of origin of all breast cancers and depending upon its genetic alterations, a given tumor arrests at a distinct stage of development. (D) Hypothesis 3: each molecular subtype originates in a normal epithelial cell along the luminal differentiation hierarchy. However, the transformation events

derive from a transformed cell at each stage, which would then form the bulk of the tumor by symmetric divisions (Hypothesis #1, Figure 7B). In this scenario, MaSCs would give rise to Claudin-low tumors, Luminal progenitor cells to Basal-like tumors, and mature luminal cells to tumor luminal A and B tumors cells, with all cells within the tumor being TICs. The potential cell of origin of HER2-enriched tumors could be an epithelial cell with an intermediate differentiated state between the luminal progenitor and the mature luminal cell, which is what the expression profile of this subtype suggests.

Although appealing in its simplicity, this hypothesis argues against other studies that have identified TICs/CSCs (i.e. CD44$^+$/CD24$^-$ cells) with mesenchymal properties in a variety of breast tumors and at a low frequency within these tumors (Creighton et al., 2009; Li et al., 2008; Fillmore and Kuperwasser, 2008; Sheridan et al., 2006). A second hypothesis is that the cell of origin for each intrinsic subtype could be the MaSC (Hypothesis #2, Figure 7C). In this scenario, transformed MaSC (with Claudin-low/mesenchymal features) would have the capacity to divide symmetrically and asymmetrically, with the asymmetric divisions resulting in cell differentiation with arrest at specific stages of differentiation depending upon the genetic events present within each particular tumor. In this context, the bulk of the tumor would be composed of either luminal progenitor/Basal-like cells, or more differentiated luminal cells, with each molecular subtype having a subpopulation of cells that are mesenchymal/Claudin-low. These undifferentiated cells within tumors would be more resistant to treatment and possibly the cells responsible for spread to distant organs. In the metastatic site, these mesenchymal/Claudin-low cells would undergo their aberrant differentiation process, which would include an apparent mesenchymal—epithelial transition (MET) as has been described in embryonic development studies (Chaffer et al., 2007). This, as well as Hypothesis #1, could explain why distant metastases display the same molecular breast cancer subtype as their primary tumors (Weigelt et al., 2005).

We have experimentally approached this second hypothesis by trying to identify epithelial tumor cells with mesenchymal characteristics across 86 samples, including 20 Claudin-low tumors. In order to do this, we used dual labeling immuno-fluorescence with a pan-keratin epithelial marker and the mesenchymal marker vimentin. Interestingly, the vast majority of dual positive samples (25/28, 85%) were observed in the Claudin-low (Figure 2A—C) and Basal-like subtypes, suggesting that these two tumor subtypes possess epithelial cells with mesenchymal features. The more differentiated subtypes (HER2-enriched, Luminal A and B) were either not enriched or the frequency of these cells was so low that we were unable to identify them. Moreover, we provided evidence that a fraction (~10%) of cells within the Basal-like BRCA1-mutated

(genetically and/or via microenvironment influences) render differentiated cells with MaSC-like features including the ability to self-renew and divide asymmetrically. Abbreviations: MaSC, mammary stem cell; MyoProg, myoepithelial progenitor; Mature-Myo, mature myoepithelial cells; LumProg, luminal progenitor; Late-LP, late luminal progenitor; Mature-L, mature luminal cells.

SUM149PT cell line were mesenchymal/Claudin-low cells that can self-renew and differentiate into Basal-like cells (Prat et al., 2010). Conversely, we could not identify Claudin-low cells within the Luminal MCF-7 cell line. Thus, this data argues in favor of the MaSC being the cell of origin of Claudin-low and Basal-like tumors (as opposed to the luminal progenitor), while the cell of origin of the more differentiated epithelial tumors (i.e. Luminal A, B and HER2-enriched) subtypes is not as clear.

A third hypothesis also exists where a molecular subtype originates in non-stem cell somewhere along the luminal differentiation hierarchy, but that the transformation event imparts self-renewal abilities and mesenchymal features, thus effectively creating a CSC from a differentiated cell (Chaffer and Weinberg, 2010; Gupta et al., 2009). In this scenario, the cells that acquired these CSC characteristics would then symmetrically and asymmetrically divide to create the mixed cell type containing tumor (Hypothesis #3, Figure 7D). An interesting twist on this hypothesis is that if, for example, a Basal-like/ luminal progenitor type cell is the primary cell type of transformation, then Claudin-low tumors may arise via EMT-inducing event that drives these rare tumors into a more mesenchymal state. Lastly, and the hypothesis we favor, some combination of these hypotheses may occur where Claudin-low and Basal-like tumors arise from transformation of MaSC (Hypothesis #2) with limited differentiation into luminal progenitors in the case of Basal-like cancers, while the formation of Luminal tumors may occur via transformation of differentiated cells (Hypothesis #1 and/or #3). Further studies are clearly required to unambiguously determine the cell of origin for each intrinsic subtype, however, studies focused on breast cancer stem cell should consider tumor subtype when interpreting results as we feel it likely that different transformation schemes, and cell types of transformation, may be occurring within different subtypes.

7.   Discussion

In this review, we have dissected the most recent data on the intrinsic classification of breast cancer with a special focus on the Claudin-low subtype. Importantly, we show and reiterate that the information provided by these molecular entities is not fully recapitulated by the classical pathological markers. We envision an integration of the intrinsic subtypes with the four main clinical treatment groups (HR+/HER2−, HR+/HER2+, HR−/HER2+ and triple-negative) in order to improve patient outcomes. In addition, other variables beyond gene expression and clinical-pathological variables, like gene mutation status or DNA copy number changes, will be needed to further stratify patients into more precise prognostic and/or specific treatment groups.

The HR+/HER2− group of tumors is mainly composed of two subtypes: Luminal A (good prognosis, chemoresistant and endocrine sensitive) and Luminal B (poor prognosis, mainly chemoresistant and endocrine less sensitive). As discussed above, a main difference between A vs. B is proliferation status, which is low in Luminal A and high in Luminal B tumors. In this context, genomic prognostic assays such as the OncoTypeDX and the NKI 70-gene signature (or even the pathological marker Ki-67) have the ability to identify tumors with high risk of recurrence, which are mainly Luminal B tumors. An important issue here will be to find which ER+ patients benefit from chemotherapy. As suggested by data from neoadjuvant clinical trials, Luminal B tumors benefit more from chemotherapy than Luminal A tumors, although only less than ∼20% of Luminal B patients eventually achieve a pCR. This increased benefit with the administration of chemotherapy in Luminal B is concordant with data coming from NSABP-B20 trial where only node-negative HR+ patients with high OncoTypeDX RS benefited from adjuvant chemotherapy (Paik et al., 2006).

In the HR+/HER2+ group of tumors, two subtypes are mainly identified: Luminal B and HER2-enriched. Here a major challenge will be to elucidate differences between the two molecular subtypes in terms of efficacy of chemotherapy, anti-hormonal therapy, and anti-HER2 therapy. For example, are HR+/HER2+/Luminal B tumors less or more sensitive to anti-HER2 therapies than HR+/HER2+/HER2-enriched tumors, and do they respond better to anti-hormonal therapies than HR+/HER2+/HER2-enriched tumors?

Within HR−/HER2+ tumors, ∼50−88% of these fall into the HER2-enriched subtype, followed by the other poor prognostic subtypes. Here the challenge will be to determine if HR−/HER2+ that are not of the HER2-enriched subtype, benefit from anti-HER2 therapies, and if HER2+ tumors that are not of the HER2-enriched subtype show similar or different response rates to trastuzumab when compared to HER2+/HER2-enriched tumors. Finally, within triple-negative disease, Basal-like and Claudin-low are the most frequent subtypes identified. Further studies focusing on the efficacy of particular chemotherapies and/or targeted therapies such as the PARP inhibitors and/or anti-CSC therapies in these subgroups of patients are warranted. It will be important to determine if Basal-like and Claudin-low tumors show similar responses to common therapies as they may given their expression similarities, or they may not given their differences including vast differences in proliferation rates. Overall, we believe that the information provided by the intrinsic subtypes, when combined with the current clinical-pathological markers, helps to further explain the biological complexity of breast cancer, should increase the efficacy of current and novel therapies, and ultimately will improve outcomes for breast cancer patients.

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